

The Effect of Pre-Extraction Preparation on Antioxidant Compounds of *Sauropus androgynus* (L.) Merr. Leaves Extracts

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ABSTRACT

Sauropus androgynus (L.) Merr. (Phyllanthaceae) is a green vegetable. It is rich in natural antioxidant compounds such as phenolics and flavonoids. The pre-extraction preparation can affect the results of extracting compounds from natural materials. This study aims to determine the effect of pre-extraction procedures, including sample form (fresh/dried), drying process (oven-drying/indirect sunlight-drying/air-drying), and particle size (ground/powdered) on total phenolic and flavonoid levels of ethanolic extracts of *S. androgynus* leaves and their antioxidant activity against DPPH radicals. *S. androgynus* leaves were extracted using the cold maceration method. The total phenolic and flavonoid contents of the extract were determined by the colorimetric method using a UV-Vis spectrophotometer. Antioxidant activity was measured based on the ability of the extracts to reduce DPPH free radicals. The results showed that the pre-extraction preparation influenced the antioxidant components obtained in *S. androgynus* leaves. According to sample form, fresh leaves contained a higher phenolics and flavonoids (33.66 mg GAE/g and 11.61 mg QE/g, respectively) than dried leaves. Whereas according to particle size, powdered dried leaves have higher phenolics and flavonoids content (45.12 mg GAE/g and 12.54 mg QE/g, respectively) than ground dried leaves. The leaves drying method that produced the highest phenolic content was oven-drying (47.08 mg GAE/g), while the one with the highest flavonoid content was air-drying (8.87 mg QE/g). All extracts had IC₅₀ values against DPPH radicals around 85.71-93.91 ppm. Pre-extraction preparation in optimum condition makes the extraction process more efficient and effective in obtaining the target compounds with antioxidant activities.

Keywords: antioxidant; flavonoids; phenolics; pre-extraction; *Sauropus androgynus*

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INTRODUCTION

Substances used in small concentrations to inhibit or reduce oxidation caused by an oxidant are known as antioxidants. One of the mechanisms of action of antioxidants to deactivate radicals is donating hydrogen atoms to free radicals to form a more stable new radicals (Singh & Singh, 2008). Plants are a source of natural ingredients with natural antioxidant potential include phenolic compounds such as phenolic acids (ferulic acid, gallic acid, ellagic acid), flavonoids (quercetin, catechins, hesperidin), tannins, anthocyanins, lignin, carotenoids (beta-carotene, lycopene, lutein), vitamins (vitamins A, C, E), etc. (Moharram & Youssef, 2014; Altemimi et al., 2017).

Green vegetables, such as *Sauropus androgynus* (L.) Merr, are a great source of antioxidants (Andarwulan et al., 2010). This plant, in Indonesia, is known as "katuk". Empirically, *S. androgynus* has been used for antipyretic, the simple evacuation of a retained placenta after childbirth, increasing and accelerating breast milk supply for breastfeeding mother, etc. (Petrus, 2013). The antioxidant capacity of *S. androgynus* leaves is related to

its various pharmacological activities has been reported, such as antimicrobial, cytotoxic against cancer cells, anti-inflammatory, and wound healing (Petrus, 2013; Khoo et al., 2015). The pharmacological activity of *S. androgynus* is related to its secondary metabolites. The leaves of *S. androgynus* contain bioactive compounds such as phenolics, tannins, flavonoids, anthocyanins, phytosterols, etc. (Petrus, 2013). Wijono (2004) reported that the phenolic compounds detected in *S. androgynus* leaves were phenolic acid, such as para-hydroxy benzoic acid, ferulic acid, caffeic acid, and vanillic acid. Djamil & Zaidan (2017) reported that the methanol extract of *S. androgynus* leaves contained flavonoids of flavonols and flavones.

The first step in studying the chemical contents of plants is to prepare plant samples for the following extraction process (Azwanida, 2015). Preparation for extraction of phenolic compounds also depends on the sample matrix and the chemical properties of the phenolic compounds (Khoddami et al., 2013). Plant samples can be fresh or dry from the whole plant or parts of plant materials such as leaves, stems, roots, etc. The dried form of the plant sample is preferred because it is more durable

(not easily damaged) and more accessible to be stored than the fresh form. In contrast, the fresh plant material may still contain more chemical compounds than the dried form. Several simple techniques for drying plant material include air-drying, oven-drying, freeze-drying (Khoddami et al., 2013), or indirect sunlight drying. Depending on the part of the plant that has to be dried, air-drying may take 3-7 days or even months. Oven-drying is a faster drying process than the air-drying and indirect-sunlight drying methods (Azwanida, 2015).

Another preparation procedure for extraction is using ground or powdered materials. Ideally, a small and homogenized sample particle size will make the extraction process runs more effectively and efficiently. It is due to the high contact between the sample and the extracting solvent. Resizing of sample particles can be done using a mortar and pestle or an electric blender (Azwanida, 2015).

This study aimed to evaluate the total phenolic and flavonoid contents in the ethanolic extracts of *S. androgynus* leaves. The leaves of *S. androgynus* were prepared under various pre-extraction conditions. In addition, the antioxidant activity of the extracts against DPPH radicals was also determined.

METHODS

Materials

Fresh green leaves of *S. androgynus* were harvested in May 2019 from Unit Konservasi Budidaya Biofarmaka (UKBB), Pusat Studi Biofarmaka Tropika LPPM IPB, Bogor, West Java, Indonesia. Plant determination was performed in the same place with the specimen number BMK0168092016. The specimen was deposited in the Laboratory of Pharmacognosy, Faculty of Pharmacy and Sciences, Universitas Muhammadiyah Prof. DR. HAMKA, Jakarta. Ethanol as solvent extraction in analytical grade was obtained from Merck (Darmstadt, Germany). Gallic acid, quercetin, and 2,2-diphenyl-1-picryl-hydrazyl (DPPH) were obtained from Sigma-Aldrich Co. (St. Louis, USA).

Preparation of Samples

The pre-extraction conditions of *S. androgynus* leaves were classified into three groups. They were given the following information:

1. The first group of samples (G1) were treated differently depending on their form, such as (a) fresh and (b) dried leaves. The fresh leaves were crushed in a mortar (diameter 160 mm). The dried leaves were obtained by drying the leaves using the air-drying method. The dried leaves were then ground using an electric blender.
2. The second group of samples (G2) was treated based

on the drying process. The leaves were dried by: (a) oven-drying using a vacuum oven at a maximum temperature of 50 °C for 1-2 days; (b) indirect sunlight by exposing the samples to the sun with a black cloth covered at 32-34 °C for three days; and (c) air-drying indoors with good air circulation at room temperature (25-27 °C) for three days. The method to measure the drying temperature was using the digital room thermometer.

3. The third group of samples (G3) was treated based on particle size. Dried leaves obtained from the drying process using the air-drying method were divided into two groups: (a) ground samples, the sample was chopped in the mortar without sieving; (b) powdered sample, the dried sample was blended and then sieved with a sieve Mesh number 40 so that the particle size is uniform with a size of 425 µm (slightly coarse powder) (Ministry of Health Republic of Indonesia, 2008).

Extraction

Each sample of *S. androgynus* leaves (150.0 g) was extracted by cold maceration using 70% ethanol as the extracting solvent following the procedure in Indonesian Herb Pharmacopoeia. Each extraction process was carried out for 24 hours with stirred occasionally. The filtrate was separated from the residue using Whatman filter paper. The residue was macerated three times. The filtrate from each solvent was collected and evaporated using a vacuum rotary evaporator N-1200 BS series (EYELA, Shanghai, China) at 50 °C and continued using a water bath at 50 °C until a crude extract was obtained (Ministry of Health Republic of Indonesia, 2008).

Characterization of the Extract

The characterization parameters of *S. androgynus* leaves extract were determined, including the organoleptic, percentage of extract yield, and total ash content according to the procedure stated in the Indonesian Herb Pharmacopoeia (Ministry of Health Republic of Indonesia, 2008). The water content was determined using a Moisture Balance Analyzer.

Phytochemical Screening

The compounds of the extracts are identified qualitatively according to Hanani (2015) and Indonesian Herb Pharmacopoeia (Ministry of Health Republic of Indonesia, 2008). The phenolics were identified using 3% FeCl₃ reagent, flavonoids using AlCl₃ reagent, and tannins using 10% gelatine reagent. The alkaloids were identified using Dragendorff, Mayer, Wagner, and Bouchardat reagents. The steroids/triterpenoids were identified using Liebermann Burchard and Salkowski reagents. Identification of saponins was carried out by frothing testing.

Determination of Total Phenolic Content (TPC)

TPC determination followed a procedure Hikmawanti et al. (2020) with modification using Folin-Ciocalteu reagent (FCR) and gallic acid as standard. Ethanolic extract of *S. androgynus* leaves in ethanol solution was piped as much as 300 μ L and then added with 1.5 mL of FCR (1:10) and homogenized. After incubated for 3 min, then 1.2 mL of 7.5% Na_2CO_3 solution was added. Finally, distilled water was added to the mixture to obtain a total volume of 10 mL. The mixture was incubated again for 60 min at room temperature. The absorbance of the solution was measured using a UV-Vis Shimadzu UV-1601 Series (Kyoto, Japan) spectrophotometer with a maximum wavelength of 756.5 nm at room temperature. The maximum wavelength was obtained through optimization using gallic acid (30 μ g/mL) in the range of 600-800 nm. The absorbance obtained is plotted into the linear regression equation. TPC is expressed in mg, which is equivalent to gallic acid per g of extract. Each extract was tested for five replication and reported as mean \pm SD.

Determination of Total Flavonoid Content (TFC)

The determination of TFC followed the procedure of Chang et al. (2002) with modification. The standard used is quercetin. Ethanolic extract of *S. androgynus* leaves in methanol solution was piped as much as 0.5 mL. Then, 1.5 mL of methanol, 0.1 mL of AlCl_3 10%, and 0.1 mL of 1 M sodium acetate were added. Finally, distilled water was added up to 5 mL. The solution was incubated for 60 min at room temperature. The absorbance of the solution was measured with a UV-Vis Shimadzu UV-1601 Series (Kyoto, Japan) spectrophotometer at a wavelength of 434 nm. The absorbance obtained is plotted into the linear regression equation. TFC is expressed in mg, which is equivalent to quercetin per g of extract. Each extract was tested for five replication and reported as mean \pm SD.

Antioxidant Activity Assay

The test for the antioxidant activity of *S. androgynus* leaves extract was carried out using the DPPH method following the procedure Wan et al. (2011) with modification. Quercetin was used as a reference. The extract was dissolved in methanol and diluted into five concentrations (20-100 ppm). Quercetin solution was dissolved in methanol and diluted to 2-10 ppm. Each concentration of the extract or quercetin solution was pipette 1 mL and added 1 mL of 0.5 mM fresh DPPH in methanol. The solution was added with methanol to a total volume of 5 mL. The mixture in a tube covered with aluminium foil paper, incubated for 30 minutes at room temperature in dark conditions. The blank solution was made from 1 mL DPPH mixed with 4 mL methanol. Furthermore, the absorption was measured at a wavelength of 515.5 nm. Each concentration series of each sample was tested triplicate. Results are reported as mean \pm SD. The calculation of the percentage of DPPH radical inhibition uses the formula:

$$\text{Inhibition of DPPH (\%)} = \frac{A_b - A_s}{A_b} \times 100$$

where A_b is the absorbance of the blank and A_s is the absorbance of the sample.

RESULTS AND DISCUSSION

The characterization of *S. androgynus* leaves extracts from various pre-extraction conditions can be seen in Table 1. The yield of extract prepared from the dry leaves (35.34%) was higher than the fresh leaves. The yield of extract prepared from the air-drying process (36.26%) showed the highest yield compared to other drying processes (air-drying > oven-drying > indirect sunlight-drying). Extracts produced from powdered (homogenized particle size) leaves had a greater extract yield (36.06%) than those made from ground leaves.

Table 1. The characteristics of *S. androgynus* leaves ethanolic extracts in various pre-extraction conditions.

Pre-extraction conditions	Percentage of extraction yield (% w/w)	Total ash (% w/w)	Water content (% w/w)
Sample Form			
Fresh	10.75 \pm 0.60	7.37 \pm 0.74	3.81 \pm 0.44
Dried	35.34 \pm 3.09	6.76 \pm 0.79	2.08 \pm 0.65
Drying Process			
Oven-drying	31.25 \pm 2.41	7.39 \pm 1.21	2.55 \pm 1.59
Indirect sunlight-drying	25.39 \pm 2.21	7.00 \pm 1.40	1.75 \pm 0.75
Air-drying	36.26 \pm 3.38	7.14 \pm 1.08	1.97 \pm 0.77
Particle Size			
Grinded	35.99 \pm 3.41	7.51 \pm 1.07	1.88 \pm 0.71
Powdered	36.06 \pm 0.75	7.94 \pm 1.30	1.97 \pm 0.56

Note: The test of each extract was carried out five times and reported in mean \pm SD.

Table 2. The phytochemical screening of *S. androgynus* leaves ethanolic extracts in various pre-extraction conditions.

Pre-extraction conditions	Compounds of <i>S. androgynus</i> Leaves Ethanolic Extracts						
	Alkaloids	Phenolics	Flavonoids	Tannins	Saponins	Steroids	Triterpenoids
Sample form							
Fresh	+	+	+	+	+	+	-
Dried	+	+	+	+	+	+	-
Drying Process							
Oven-drying	+	+	+	+	+	+	-
Indirect sunlight-drying	+	+	+	+	+	+	-
Air-drying	+	+	+	+	+	+	-
Particle Size							
Grinded	+	+	+	+	+	+	-
Powdered	+	+	+	+	+	+	-

Note: (+) = detected; (-) = not detected

Based on the results in Table 1, the ash content produced by all extracts was around 6.76-7.94%. Ash content is determined to provide an overview of mineral content derived from plants or from outside of plants (Departemen Kesehatan RI, 2000). The water content of each extract was 1.75-3.81%. The percentage of water content is determined to provide an overview of the maximum limit for water contained in the extract, especially for materials that absorb moisture quickly. The high water content in an extract is a good condition for microbes to grow (World Health Organization, 1998). Determination using a moisture analyzer is an efficient way of measuring with many samples in a short analysis time.

All extracts of *S. androgynus* leaves showed the presence of alkaloid, phenolic, flavonoid, tannin, saponin, and steroid compounds (Table 2). Ethanol is a solvent capable of dissolving polyphenols, tannins, polyacetylene, flavonols, terpenoids, sterols, and alkaloids (Azmir et al., 2013; Pandey & Tripathi, 2014). Simple phenolic compounds, polyphenols, and flavonoids are generally extracted with alcohol (methanol, ethanol, and propanol) solvents, water, acetone, and ethyl acetate (Stalikas, 2007). The amount of phenolic compounds and their derivatives extracted in a solvent depends on the type and chemical properties of the phenolic contained in the extracted plant material (Khoddami et al., 2013). In this study, terpenoids were not detected in all extracts. In contrast, Fikri & Purnama (2020) were reported that the leaves of *S. androgynus* contained terpenoids. In addition, another study by Awaludin et al., (2020) reported that ethanolic extract of *S. androgynus* leaves contained phytol, a terpenoid, which was at a level of 4.03%. Phytochemical screening with a simple method that used in this study has weaknesses. For example, false negative reactions can occur because the presence

of compounds identified in the test solution from the extract is too small so it cannot be detected with the reagents used.

The determination of total phenolic and flavonoid contents of *S. androgynus* leaves was carried out by a colorimetric method using a UV-Vis spectrophotometer. Phenolic is the largest compound present in nature, often found in plants. Several ways were used to determine the phenolic content of plant extracts, including Folin-Denis, Folin-Ciocalteu, permanganate titration, colorimetry with iron salts, or UV absorption. However, Folin-Ciocalteu is the most popular method because it is simple and reproducible. This method relies on the transfer of electrons from the alkaline medium of the phenolic compound to the phosphomolybdate/ phosphotungstate to form a blue complex ($(\text{PMoW}_{11}\text{O}_{40})^{4-}$) which is determined using a spectrophotometer at about 760 nm. The total phenolic content was then equated with mg of gallic acid per gram or kilograms or liters of extract (Dai & Mumper, 2010).

Flavonoids are phenolic derivatives with a conjugated aromatic ring system so they can provide absorption in UV rays. As a standard, quercetin is one of the flavonol groups with a hydroxyl group that is substituted on carbon number 5 and 7 in the aromatic ring A and carbon number 3 in C heterocyclic, and a ketone group substituted on carbon number 4 in the heterocyclic C ring. Hydroxyl on carbon number 3 or 5 and ketone at carbon position number 4 is made a complex with AlCl_3 will form a bright yellow complex that is stable in visible light (Hanani, 2015).

The concentration range of gallic acid used to determine the standard curve for gallic acid was 18-66 ppm.

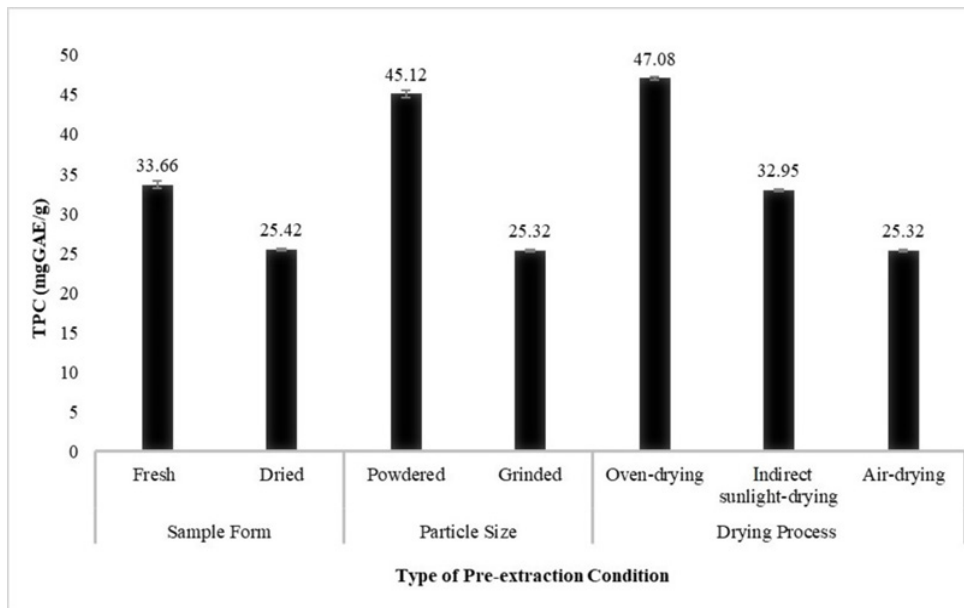


Figure 1. Total phenolic content (TPC) of *S. androgynus* leaves ethanolic extracts in various pre-extraction conditions. The test of each extract was carried out five times and reported as mean \pm SD.

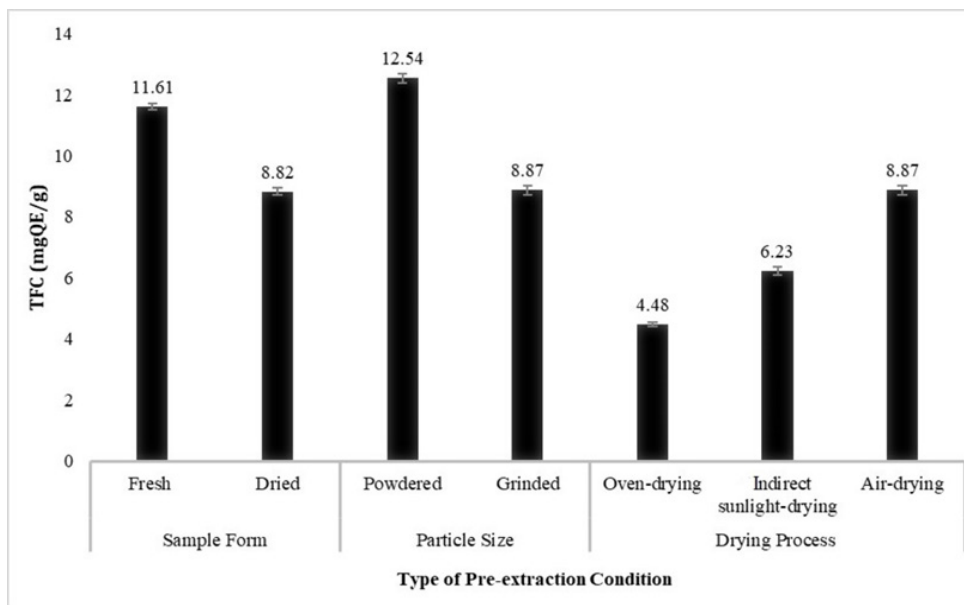


Figure 2. Total flavonoid content (TFC) of *S. androgynus* leaves ethanolic extracts in various pre-extraction conditions. The test of each extract was carried out five times and reported as mean \pm SD.

The gallic acid calibration curve produced a linear regression equation $y = 0.0093x + 0.1824$ with $r = 0.99$. Meanwhile, the concentration range for determining the standard quercetin curve was 33-129 ppm. Quercetin calibration curve produced a linear regression equation $y = 0.0042x + 0.1897$ with a value of $r = 0.99$. The value of r approaching number 1 indicated the linear line equation. The total phenolic and flavonoid contents of *S. androgynus* leaves extract under various conditions of pre-extraction preparation were presented in Figure 1 and Figure 2. Based on these results, the extract prepared

from fresh leaves contained phenolic and flavonoids higher than dry ones. According to Suvarnakuta et al. (2011), the drying process of plant materials can affect the degradation of xanthones (chemical compounds in mangosteen rind) and their antioxidant activity. However, dry plant material has the advantage. It can be stored for a long time before being used in different processes, such as extraction. The drying process can inhibit the degradation of compounds due to enzymatic and microbial growth during storage (Pinela et al., 2012).

Table 3. Antioxidant activities of *S. androgynus* leaves extracts in various pre-extraction conditions.

Pre-extraction Conditions		IC ₅₀ value of <i>S. androgynus</i> Ethanolic Extract (ppm)
Sample Form	Fresh	87.04 ± 0.51
	Dried	90.65 ± 0.41
	Oven-drying	85.71 ± 2.06
Drying Process	Air-drying	90.65 ± 0.41
	Indirect sunlight-drying	93.91 ± 1.57
	Grinded	90.65 ± 0.41
Particle Size	Powdered	89.82 ± 3.24
Quercetin as a reference		8.92 ± 0.72

Note: The test of each extract was carried out five times and reported in mean ± SD.

The extract prepared from the powdered plant materials (homogenized particle size) contains higher phenolic and flavonoid levels than the ground ones. Extract prepared from dried leaves by the oven-drying method contained higher phenolic content than other drying processes (oven-drying > indirect sunlight-drying > air-drying). In contrast, extract prepared from dried leaves by air-drying had the highest levels of flavonoids compared to other drying processes (air-drying > indirect sunlight-drying > oven-drying). This result suggested that the selection of the pre-extraction process affects the levels of antioxidant compounds (phenolic and flavonoids) in the extract. The efficiency of the drying process of plant material is influenced by the temperature used in the drying method. The oven-drying method in this study had a shorter drying time than other methods. This is advantageous because enzymes that degrade polyphenolic compounds, such as polyphenol oxidase, can be deactivated immediately (Rababah et al., 2015). Thus, in this study, the total phenolic content in the extract produced from dried leaves with the oven-drying process had the highest concentration compared to other methods. However, the levels of flavonoids in the extract obtained from dried leaves with the oven-drying process were lower than those with air-drying. The heat used in the oven-drying process can also cause a decrease in the chemical contents (Yuan et al., 2015).

The data in Table 3 showed the antioxidant potential of 70% ethanol extract of *S. androgynus* leaves with variations in pre-extraction conditions. The IC₅₀ value of the ethanolic extract of *S. androgynus* leaves was 85.71-93.91 ppm. It means that the ethanolic extract is a potent source of antioxidants (IC₅₀ = 50-100 ppm). DPPH method is fast, simple, and relatively more inexpensive, reproducible than other radical scavenging in vitro assays

(Alam et al., 2013). Nonetheless, the DPPH radical interacts with other radicals (alkyl) and is sensitive to some Lewis bases. In addition, this method also requires an organic solvent in the test. The presence of oxygen can also react with DPPH directly in conditions without light protection, which causes a decrease in absorption (Singh & Singh, 2008). Evaluation of the antioxidant potential of a sample using this method is based on changes in DPPH free radical absorption after reacting with an antioxidant substrate that can donate a hydrogen atom. This change can be observed from the decrease in the intensity of the dark purple color of the DPPH radical measured using a spectrophotometer at a wavelength in the range 515-517 nm (Alam et al., 2013; Prior et al., 2005).

There are two antioxidant mechanisms of phenolic compounds: (a) the ability of phenol functional groups to donate hydrogen atoms to free radicals (hydrogen-atom transfer, HAT) and (b) single-electron transfer (SET) from phenolic antioxidants (ArOH) to free radicals (R' by forming radical cations (ArOH⁺) (Quideau et al., 2011). Flavonoids have several mechanisms of action as antioxidants, one of them is direct radical reduction. Flavonoids will be oxidized by radicals, resulting in more stable (less reactive) radicals. Otherwise, flavonoids stabilize reactive oxygen species by neutralizing the radicals because of the high reactivity of the hydroxyl groups of flavonoids (Nijveldt et al., 2001).

CONCLUSION

The pre-extraction preparation process, such as sample form, drying process, and particle size, affects the yield of antioxidant compounds, including phenolics and flavonoids in the *S. androgynus* leaves extract. The pre-

extraction procedures need to be prepared in optimum condition to efficiently and effectively extract the target compounds.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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